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Structural and functional analysis of aa_3-type and cbb_3-type cytochrome *c* oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design

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Summary

In *Paracoccus denitrificans* the aa_3-type cytochrome *c* oxidase and the bb_3-type quinol oxidase have previously been characterized in detail, both biochemically and genetically. Here we report on the isolation of a genomic locus that harbours the gene cluster *ccoNOQP*, and demonstrate that it encodes an alternative cbb_3-type cytochrome *c* oxidase. This oxidase has previously been shown to be specifically induced at low oxygen tensions, suggesting that its expression is controlled by an oxygen-sensing mechanism. This view is corroborated by the observation that the *ccoNOQP* gene cluster is preceded by a gene that encodes an FNR homologue and that its promoter region contains an FNR-binding motif. Biochemical and physiological analyses of a set of oxidase mutants revealed that, at least under the conditions tested, cytochromes aa_3, bb_3 and cbb_3 make up the complete set of terminal oxidases in *P. denitrificans*. Proton-translocation measurements of these oxidase mutants indicate that all three oxidase types have the capacity to pump protons. Previously, however, we have reported decreased H^+/e^- coupling efficiencies of the cbb_3-type oxidase under certain conditions. Sequence alignment suggests that many residues that have been proposed to constitute the chemical and pumped proton channels in cytochrome aa_3 (and probably also in cytochrome bb_3) are not conserved in cytochrome cbb_3. It is concluded that the design of the proton pump in cytochrome cbb_3 differs significantly from that in the other oxidase types.

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Introduction

In mitochondrial respiration the reduction of oxygen to water is catalysed by the aa_3-type cytochrome *c* oxidase. This membrane-bound protein complex is the last component of a linear respiratory pathway in which electrons from NADH and succinate are transferred, in a sequence of redox reactions, to oxygen. The aerobic respiration in bacteria, on the other hand, often proceeds via a more complex electron-transfer network. Alternative respiratory pathways enable a bacterium to adjust the composition and/or the efficiency of oxidative phosphorylation in response to changes in environmental conditions (Anraku and Gennis, 1987; van der Oost *et al.*, 1994; Garcia-Horsman *et al.*, 1994a; van Spanning *et al.*, 1995a).

In addition to a number of alternative quinone-reducing dehydrogenases, the terminal oxidases appear to represent an important site of flexibility in respiratory networks. To date, the best-characterized bacterial respiratory system is the one in *Escherichia coli*. In this bacterium two distinct quinol oxidases (cytochromes bo_3 and *bd*) are expressed during aerobic growth, but a cytochrome *c* branch is absent (Anraku and Gennis, 1987). *Paracoccus denitrificans*, in turn, possesses three distinct terminal oxidases. An aa_3-type cytochrome *c* oxidase (Raitio *et al.*, 1987; 1990; Steinrücke *et al.*, 1987; van Spanning *et al.*, 1990; van der Oost *et al.*, 1991), as well as a bb_3/ba_3-type quinol oxidase, hereafter called cytochrome bb_3, have been characterized previously (de Gier *et al.*, 1994; Richter *et al.*, 1994). In addition, the expression of an alternative cytochrome *c* oxidase has been suggested (Bosma, 1989; de Gier *et al.*, 1992; 1994; Raitio and Wikström, 1994).

In the present study, a *P. denitrificans* $\Delta aa_3/bb_3$ mutant

was used for the purification of an alternative cytochrome *c* oxidase: cytochrome cbb_3. Using the N-terminal amino acid sequence of one of its subunits, a genomic locus, *ccoNOQP*, was isolated that contained a gene cluster with four open reading frames. Sequence analysis of the flanking regions revealed the presence of a *ccoGH* cluster downstream of *ccoNOQP*. In the upstream region two open reading frames were found, the derived sequences of which are homologous to HemN and FNR.

Analysis of the oxygen consumption by single and multiple oxidase mutants of *P. denitrificans* suggests that, besides cytochrome aa_3 and cbb_3, no additional cytochrome *c* oxidase is present. The currently generated set of oxidase mutants of *P. denitrificans* was used to demonstrate that all three terminal oxidases have the capacity to pump protons. It is discussed, however, that in the cbb_3-type cytochrome *c* oxidase the coupling between oxygen reduction and proton translocation may be less tight than in cytochromes aa_3 and bb_3/bo_3.

Results

Purification of the cbb$_3$*-type cytochrome* c *oxidase*

In a previous study, the simultaneous deletion of cytochromes aa_3 and bb_3 in *P. denitrificans* ($\Delta aa_3/bb_3$) has been reported. Analysis of this mutant revealed (i) the presence of an alternative cytochrome *c* oxidase, (ii) the presence of only protohaem IX (haem B) in membrane extracts, and (iii) an increased expression of cytochromes *c* and *b*. No quinol oxidase activity was detectable in this mutant (de Gier *et al.*, 1994). Here, we report the isolation of a *cb*-type cytochrome *c* oxidase from membranes of the *Paracoccus* $\Delta aa_3/bb_3$ mutant. Both the activity and the subunit composition of the oxidase complex turned out to be rather unstable during the purification, as described in the *Experimental procedures*. As will be discussed below, a cytochrome *c* subunit with an apparent molecular mass of 45 kDa is lost during the purification of *Paracoccus* cytochrome cbb_3 (as described in the *Experimental procedures*).

Analysis of the purified cytochrome *c* oxidase by a Coomassie brilliant blue-stained SDS–PAGE gel indicates that it consists of two components with apparent molecular masses of 45 kDa and 30 kDa (Fig. 1A). Only the fast-migrating component has been identified as a cytochrome *c* because of the presence of covalently bound haem, as demonstrated by haem-stained SDS–PAGE (not shown). Sequencing of this 30 kDa subunit revealed the N-terminal sequence AILEKHKVLEKNATLLLVFSFLVVRI. The optical spectrum of this oxidase preparation indicates that it involves a *cb*-type cytochrome (Fig. 1B). However, the relatively small amount of cytochrome *c* in this spectrum relative to a previous preparation (de Gier *et al.*, 1994) can best be explained as the loss of a second haem

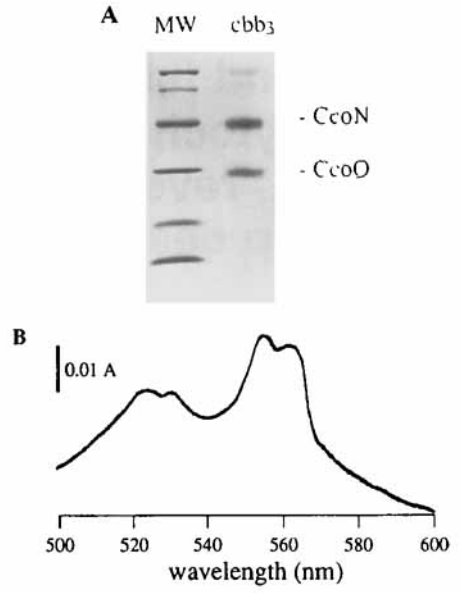

Fig. 1. A. SDS–PAGE of purified cytochrome cbb_3 (approx. 7.5 μg of protein). The molecular mass standards are: 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa.
B. Absorption spectrum of purified cytochrome cbb_3 after reduction with Na-dithionite (approx. 0.01 mg protein ml^{-1}).

C-containing subunit which would co-migrate with the 45 kDa polypeptide, as demonstrated below.

Isolation and characterization of the ccoNOQP *cluster*

Based on the N-terminal sequence of the 30 kDa subunit, degenerate primers were designed (see the *Experimental procedures*, primers 171 and 172) and used to perform the polymerase chain reaction (PCR) on genomic DNA from the $\Delta aa_3/bb_3$ mutant. A PCR product of the expected size (0.1 kb) was obtained. Sequence analysis of the PCR product indicated that the DNA sequence corresponded to the N-terminal peptide of the 30 kDa subunit (not shown). With the PCR product as a probe, the complete *ccoNOQP* cluster could be cloned (Fig. 2A). Fragments were subcloned into M13mp18/19 for sequence analysis. The complete DNA sequence has been submitted to the GenBank database Data Library (Accession Number BankIt12589 U34353).

Four open reading frames (ORFs) in the isolated genomic locus share a high degree of homology with the *ccoNOQP* gene cluster from *Rhodobacter capsulatus* (Thöny-Meyer *et al.*, 1994) and to the *fixNOQP* operons of *Rhizobium meliloti* (Kahn *et al.*, 1993; D. Kahn, 1993, EMBL Data Library Accession No. 221854), *Bradyrhizobium japonicum* (Preisig *et al.*, 1993; Fig. 3) and *Azorhizobium caulinodans* (Mandon *et al.*, 1994). As *P. denitrificans* is not known to be able to fix molecular nitrogen, the *ccoNOQP* rather than the *fixNOQP* nomenclature

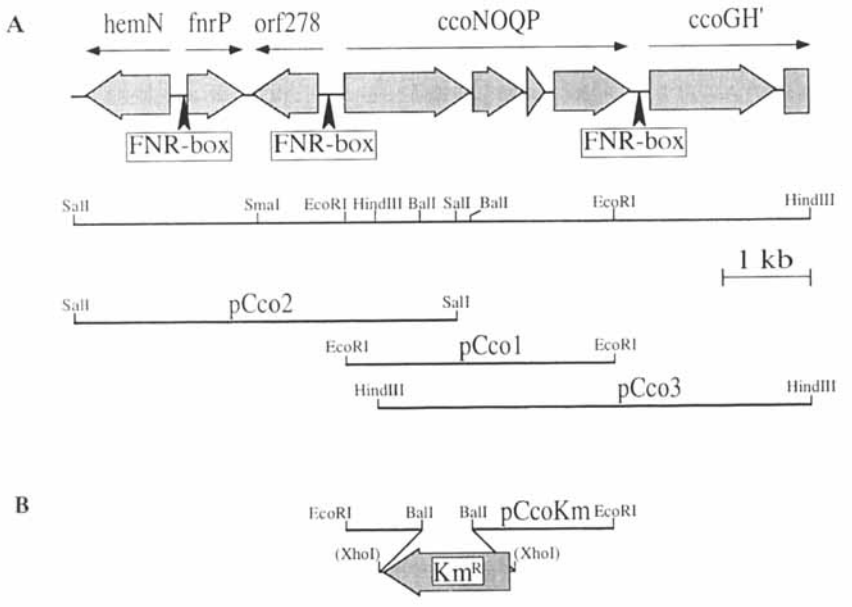

Fig. 2. A. Genetic organization and physical map of the *P. denitrificans ccoNOQP* region. Arrows represent individual genes. The positions of FNR-boxes are indicated by arrows.
B. Insertion mutants have been generated by substituting part of the *ccoNOQP* operon with a kanamycin-resistance (Km^R) cassette, deleting the 3′ half of *ccoN* and the two 5′ codons of *ccoO*.

has been adopted for these genes. The four ORFs have a GC-rich codon preference which is typical of *P. denitrificans*. The *ccoNOQP* genes are tightly clustered, with the exception of the *ccoQ* and *ccoP* genes which are 41 bases apart. As no transcription-termination signal was found here, the four genes are probably transcribed as a single operon, as has been demonstrated for *R. meliloti* (Batut *et al.*, 1989). The proposed initiation codons of all four genes are six to eight bases downstream of putative Shine–Dalgarno sequences. A putative anaerobox (TTGAC-N_4-ATCAA) is located upstream of *ccoN*. Its sequence is very similar to the binding sites of FNR in *E. coli* and of FixK in *R. meliloti* and *B. japonicum* (TTGAT-N_4-ATCAA; Spiro, 1994), and identical to the anaerobox found upstream of the *A. caulinodans fixNOQP* operon (Mandon *et al.*, 1994).

Attempts to sequence the N-terminus of subunit I (CcoN, apparent molecular mass 45 kDa (Fig. 1A)) were unsuccessful, probably because of blocking of the first amino group. The N-terminal methionine of CcoN (Fig. 3A) has been chosen because the corresponding ATG codon is downstream of a Shine–Dalgarno sequence, and because no alternative in-frame initiation codons are present. Moreover, the size of the gene correlates with that of *ccoN/fixN* in the aforementioned bacteria. The putative anaerobox is located at positions −110 and −124 of the chosen start codon. Based on sequence alignment with the subunits I of the well-characterized cytochromes aa_3 from *Rhodobacter sphaeroides* and *P. denitrificans*, as well as with the subunit I of cytochrome bo_3 from *E. coli* (Hosler *et al.*, 1993), four histidines in the CcoN sequence (H271, H321, H322 and H409) are the most probable ligands of the binuclear catalytic centre formed by a penta co-ordinated haem B and a copper atom, Cu_B (Fig. 3A). The ligands of the hexa co-ordinated haem B are H411 and (probably) H122 in the *Paracoccus* CcoN (Fig. 3A).

CcoO has an N-terminal amino acid sequence that corresponds to the N-terminal sequence determined for the 30 kDa subunit (Fig. 3B). Apart from this hydrophobic, putative membrane-spanning fragment, CcoO contains a hydrophilic, probably periplasmic, domain with a Cx_2CHx_nM motif which is the consensus for haem C binding (Fig. 3B). Apart from this motif, CcoO/FixO does not share much similarity with the available cytochrome *c* sequences (Moore and Pettigrew, 1990). CcoQ is a small protein with one hydrophobic region (Fig. 3C); apart from CcoQ and FixQ, no significant homologues have been found in sequence databases. CcoP appears to be a membrane-anchored di-haem cytochrome *c*. Its C-terminal hydrophilic domain, probably facing the periplasmic side of the plasma membrane, contains two motifs ($Gx_3Fx_3Cx_2CHx_nM$) that are well conserved among soluble mono-haem cytochromes of photosynthetic bacteria and eucaryotic algae (Mandon *et al.*, 1994). In contrast to the FixP/CcoP sequences from other sources, an N-terminal hydrophilic extension is present in the *Paracoccus* sequence (Fig. 3D), probably located at the cytoplasmic side of the membrane.

Flanking regions

Upstream of the *ccoNOQP* cluster of *P. denitrificans* is an ORF that potentially codes for a polypeptide of 278 amino acids (orf278 in Fig. 2A). It shares a rather high degree of homology (28% identical amino acids, not shown) with orf277, which is located adjacent to the *fixNOQP* operon

A

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Pd CcoN   1  --------MLDTIKLIALGTIAVLAAIAANYARPDDLAYLVNALIIMLAAGIMFLRVLRQMGNEQPA
Bj FixN   1  MSQPSISKSMTIGESGLAVVFAATAFLCVIAAAKALDAPFAFHAALSAAASVAAVFCIVNRYFERPA
Pd.CtaD   1  ------------------------------------------------------------MADAAVH
Ec CyoB   1  ---------------------------------MFGKLSLDAVPFHEPIVMVTIAGIILGGLALVG

                            TM I
Pd CcoN  60  LEPHPETQYMDDVVR----AGVIATAFWGVVGFLVGVVIAFQLAFPALNLSD--------------
Bj FixN  68  ALPPAEINGRPNYNMGPIKFSSFMAMFWGIAGFLVGLIIASQLAWPALNFDL--------------
Pd CtaD   8  GHGDHHDTRGFFTRWFMSTNHKDIGILYLFTAGIVGLISVCFTVYMRMELQHPGVQYMCLEGARLIA
Ec CyoB  34  LITYFGKWTYLWKEWLTSVDHKRLGIMYIIVAIVMLLRGFADAIMMRSQQAL-------------A

                      TM II                                          TM III
Pd CcoN 104  -----ITMGYTNFGKLRPLHTSAVIFAFGGNGLIATSFYVVQRTSAAR------LWGGNAAWFVFWG
Bj FixN 116  --------PWISFGRLRPLHTSAVIFAVGGNVLIATSFYVVQKSCRVR------LAGDLAPWFVVVG
Pd CtaD  75  DASAECTPNGHLWNVMITYHGVLMMFFVVIPALFGGFGNYFMPLHIGAPDMAFPRLNNLSYWMYVCG
Ec CyoB  87  SAGEAGFLPPHHYDQIFTAHGVIMIFFVAMPFVIG-LMNLVVPLQIGARDVAFPFLNNLSFWFTVVG
                               H                  N          D       N
                               94                 113        124     131
                                                  TM IV
Pd CcoN 164  YQLFIVLA-----------ATGYILGATQSK------EYAEPEWYVDWWLTVVWVVYLAVFLGTILK
Bj FixN 173  YNFFILVA-----------GTGYLLGVTQSK------EYAEPEWYADLWLTIVWVVYLLVFLATIIK
Pd CtaD 142  VALGVASLLAPGGNDQMGSGVGWVLYPPLST--TEAGYSMDLAIFAVHVSGASSILGAINIITTFLN
Ec CyoB 153  VILVNVSLGVGE-----FAQTGWLAYPPLSGIEYSPGVGVDYWIWSLQLSGIGTTLTGINFFVTILK
                                                                    N    T
                                                                    199  203
                      TM V                                          TM VI
Pd CcoN 214  RKEP-HIYVANWFYLSFIVTIAMLHIVNNLAIPVSLFGSKSVQLF----------SGVQDAMTQWW
Bj FixN 223  RKEP-HIFVANWFYLAFIVTIAVLHLGNNPALPVSAFGSKSYVAW----------GGIQDAMFQWW
Pd CtaD 207  MRAPGMTLFKVPLFAWSVFITAWLILLSLPVLAGAITMLLMDRNFGTQFFDPAGGGDPVLYQHILWF
Ec CyoB 216  MRAPGMTMFKMPVFTWASLCANVLIIASFPILTVTVALLTLDRYLGTHFFTNDMGGNMMMYINLIWA

                                        TM VII                                  TM VIII
Pd CcoN 269  YGHNAVGFFLTAGFLGMMYYFIPKQAERPVYSYKLSIIHFWALIFLYIWAGPHHLHYTALPDWASTL
Bj FixN 278  YGHNAVGFFLTAGFLAIMYYFIPKRAERPIYSYRLSIIHFWALIFLYIWAGPHHLHYTALPDWTQTL
Pd CtaD 274  FGHPEVYIIILPGFGIISHVISTF-AKKPIFGYLPMVLAMAAIGILGFVVWAHHMYTAGMSLTQQAY
Ec CyoB 282  WGHPEVYILILPVFGVFSEIAATF-SRKRLFGYTSLVWATVCITVLSFIVWLHHFFTMGAGANVNAF
               H E Y            S                                    HH
               276 278 280      291                                  325 326
                                                  TM IX
Pd CcoN 336  GMVFSIILWMPSWGGMINGLMTLSGAWDKLRTDPIIRMMVVAVGFYGMATFEGPMMSIKAVNFVSHY
Bj FixN 345  GMTFSIMLWMPSWGGMINGLMTLSGAWDKLRTDPVLRMLVVSVAFYGMSTFEGPMMSIKVVNSLSHY
Pd CtaD 340  FMLATMTIAVPTGIKVFSWIATMWGGSIEFKTP--MLWAFGFLFLFTVGGVTGVVLSQAPLDRVYHD
Ec CyoB 348  FGITTMIIALPTGVKIFNWLFTMYQGRIVFHSA--MLWTIGFIVTFSVGGMTGVLLAVPGADFVLHN
                 T       T  K                                                L      D
                 344     351 354                                              393    399
               TM X                                          TM XI
Pd CcoN 403  TDWTIGHVHSGALGWNGMITFGALYYLVPRLWGR-ERLYSTGLVSWHFWLATIGLVLYAASMWVSGI
Bj FixN 412  TDWTIGHVHSGALGWVGFVSFGALYCLVPWAWNR-KGLYSLKLVNWHFWVATLGIVLYISAMWVSGI
Pd CtaD 405  TYYVVAHFHYVMSLGAVFGIFAGVYY---WIGKMSGRQYPEWAGQLHFWMMFIGSNLIFFPQHFLGR
Ec CyoB 413  SLFLIAHFHNVIIGGVVFGCFAHMTY---WWPKAFGFKLNETWGKRAFWFWIIGFFVAFMPLYALGF
                   H H
                   411 413
                                  TM XII
Pd CcoN 469  MEGLMWREVDAQGFLVNAFADTVAAKFPMNVVRALGGVLYLFFALIMCYNLWATVAKQPKTQSTAAA
Bj FixN 478  LQGLMWRAYTSLGFLEYSFIETVEAMHPFYIIRAAGGGLFLIGALIMAYNLWMTVRVGEAEVQMPVA
Pd CtaD 469  Q-GMPRRYIDYPVE-----FAYWNNISSIGAYISFASFLFFIGIVFYTLFAGKRVNVPNYWNEHADT
Ec CyoB 477  M-GMTRRLSQQIDP----QFHTMLMIAASGAVLIALGILCLVIQMYVSIRDRDQNRDLTGDPWGGRT


Pd CcoN 536  VPAE
Bj FixN 545  LQPAE
Pd CtaD 530  LEWTLPSPPPEHTFETLPKREDWDRAHAH
Ec CyoB 539  LEWATSSPPPFYNFAVVPHVHERDAFWEMKEKGEAYKKPDHYEEIHMPKNSGAGIVIAAFSTIFGFA
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B

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Pd CcoO     1  MAILEKHKVLEKNATLLLVFSFLVVTIGGIVEIAPLFYLQNTIE  44
Bj FixO     1  MSFWTRHQVFEKNSIILIVGILLVIAIGGLVEITPLFYLKSTIE  44

Pd CcoO    45  KVQGMRPYTPLELKGRDIYVREGCYVCHSQMIRPM-RDEVERYG  87
Bj FixO    45  KVDGVRPYTPLELAGRNVYVREGCYLCHSQMIRPLRDEV-ERYG  87

Pd CcoO    88  HYSLAAESMYDHPFQWGSKRTGPDLARVGGRYSDEWHLDHLVDP  131
Bj FixO    88  HFSLAAESMFDHPFQWGSKRTGPDLARVGAKYSDDWHVTHLTNP  131

Pd CcoO   132  QAVVPESIMPKYGF-LLNRQVDASNMQQRLKTDALG-GVPYDDA  173
Bj FixO   132  RAIVPQSVMPGYPFLSATEVDPDTIADHMRTLRTVGVPYTDDQI  175

Pd CcoO   174  MIAAAGEDFRVQAAPDADASGLEERYPG-AQQRNFDRRPG-VSE  215
Bj FixO   176  ANASADLKAQADPDNAGADAFNKRYAKAVVRNFD-GKTGTP-TE  217

Pd CcoO   216  MDALIAYLQVLGTMVDFSTFEPDPNR                    241
Bj FixO   218  MDALIAYLQMLGTLVDFKIYNEKANLR                   244
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C

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Pd CcoQ     1  MDRYSFLRELADS----WVLLLLVVFFLGTIVFAFRPGFAAAAS  40
Bj FixQ     1  MKAILTLDNLASGLVTTIWTPVFVAIFLAIIAYAFWPRNKAAFD  44

Pd CcoQ    41  RRGRKHLP                                      48
Bj FixQ    45  EAAHLPLREE                                    54
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D

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Pd CcoP     1  MADTDDEHASPQNPDNRIELERQAADEAHKAKILAHPPEAGGDP  44
Bj FixP     1  MTDHSEFDS---------------------------------     9

Pd CcoP    45  LHPPVTPRPGATRVVRDRKGGRRVVEVPSTGHSWDGIEEYDNPL  88
Bj FixP    10  ----------------------VSGKTTTGHEWDGIKELNTPL  30

Pd CcoP    89  PRWWLWTFYATIVWGVLYLIAYPAIPLVNGATQGLLGQNYRSDV  132
Bj FixP    31  PRWWVICFYLTIVWAIGYWIVYPAWPLISSNTTGLFGYSSRADV  74

Pd CcoP   133  AAEIQRFNEANAPIQAKLVETPLEEIAADPELANYTANAGAAIF  176
Bj FixP    75  AVELANLEKIRGDKMAALGAASLADVEKDPALLALARAKGKTVF  118

Pd CcoP   177  RTWCAQCHGSGAGGATGYPSLLDNDWLWGGTLEEIHTTVMHGIR  220
Bj FixP   119  GDNCAPCHGSGGAGAKGFPNLNDDDWLWGGTLDQIMQTIQFGAR  162

Pd CcoP   221  DPKDADTRYSEMPRFGIDGLLENAQISQVVNHVLELGGLPHDAA  264
Bj FixP   163  SGHAKTHEGQMLAFGKDGVLKGDEIVTVANYVRSLSGLPTRKGY  206

Pd CcoP   265  LAAEGVEVFADNCSSCHAEDGTGDRAQGAPDLTDAVWLYGSDPA  308
Bj FixP   207  DAAKGEKIFVENCVACHGDGGKGNQEMGAPNLTDKIWLYGSDEA  250

Pd CcoP   309  TITRIVRDGPFGVMPAWTGRLSEADIVAVAAYVHSLGGGE      348
Bj FixP   251  ALIETISQGRAGVMPAWEGRLDPSTIKAMAVYVHSLGGGK      290
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Fig. 3. Alignments of the *P. denitrificans ccoNOQP*-encoded polypeptides.
A. CcoN of *P. denitrificans* (cytochrome *cbb*$_3$), FixN of *B. japonicum* (cytochrome *cbb*$_3$; Preisig *et al.*, 1993), CtaDII of *P. denitrificans* (cytochrome *aa*$_3$; Raitio *et al.*, 1990), and CyoB of *E. coli* (cytochrome *bo*$_3$; Chepuri *et al.*, 1990). Transmembrane helices are shaded and conserved residues discussed in the text are indicated.
B. CcoO of *P. denitrificans* and FixO of *B. japonicum* (Preisig *et al.*, 1993). Identical residues are boxed.
C. CcoQ of *P. denitrificans* and FixQ of *B. japonicum* (Preisig *et al.*, 1993). Identical residues are boxed.
D. CcoP of *P. denitrificans* and FixP of *B. japonicum* (Preisig *et al.*, 1993). Identical residues are boxed.

of *B. japonicum*. No homologue has yet been reported in other members of the Rhizobiaceae family. As no mutants are available, a link between this gene product and cytochrome cbb_3 remains to be demonstrated. Further upstream, two genes are located that encode homologues of *E. coli* HemN and FNR (Fig. 2A). In the latter bacterium both polypeptides play an important role during oxygen limitation: HemN catalyses an oxygen-independent bypass in haem biosynthesis (Plunkett *et al.*, 1993), and FNR is a transcription activator of genes involved in anaerobic metabolism (Spiro and Guest, 1990).

Without exception, the gene cluster *fixGHIS* is located downstream of the *fixNOQP* genes of the Rhizobiaceae family (Kahn *et al.*, 1993; Preisig *et al.*, 1993; Mandon *et al.*, 1994) and *ccoNOQP* genes of *R. capsulatus* (Thöny-Meyer *et al.*, 1994). In *R. meliloti,* a number of transposon insertions in *fixGHI* have been isolated which had a 'fix-minus' phenotype (Kahn *et al.*, 1989). Also, in *Paracoccus* at least part of this cluster is located adjacent to the *ccoNOQP* genes. Two genes homologous to *fixG* and *fixH* (Fig. 2A) have been found downstream of *ccoNOQP* but, for the reasons given above, these genes are designated *ccoG* and *ccoH*. In all instances an anaerobox is located in the *fixG* and in the *ccoG* promoter regions (Fig. 2A), suggesting expression of this oxidase type at low oxygen tensions. The *ccoG* product is a ferredoxin-like protein, with two typical 4Fe4S-binding motifs. FixGHIS has been suggested to be a polypeptide complex consisting of, at least, a redox protein (FixG) and a cation pump (FixI) (Kahn *et al.*, 1989).

Mutagenesis of ccoNO

Insertion mutants were generated by substituting part of the *ccoNOQP* operon by a kanamycin-resistance (Km^R) cassette, deleting the 3′ half of *ccoN* and the two 5′ codons of *ccoO* (Fig. 2B). The *ccoNOQP* operon was deleted in wild-type *Paracoccus* (Pd1222) and in the *ctaDI/ctaDII* double mutant Pd92.20 (Δaa_3), yielding Pd27.21 (Δcbb_3) and Pd93.12 ($\Delta aa_3/cbb_3$), respectively. Southern blot analysis of chromosomal DNA of selected Km^R clones confirmed the appropriate recombination (not shown). Analysis of the mutant strains generated, grown on minimal medium with succinate, did not show significant spectroscopic changes relative to their respective parent strain. SDS–PAGE and subsequent haem staining of cytoplasmic membranes showed that the 30 kDa cytochrome *c* is absent, and the 45 kDa cytochrome *c* is only just detectable in the Δcbb_3 mutant strains (Fig. 4).

Oxygen consumption

The oxygen-consumption rates in cell suspensions of wild type and oxidase mutants were measured polarographically, with endogenous as well as exogenous substrates (succinate or ascorbate with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD)). The oxidation rates are shown in Table 1. In separate assays the electron flow to oxygen via cytochrome *c* reductase (the bc_1 complex) was inhibited by addition of either antimycin A or myxothiazol. The endogenous respiration of the wild type was clearly affected by these inhibitors. In contrast, the endogenous respiration of the Δaa_3 mutant and the Δcbb_3 mutant was only slightly inhibited, and that of the $\Delta aa_3/cbb_3$ mutant strain appeared to be unaffected.

The effect of the cytochrome bc_1 inhibitors on succinate oxidation in the Δcbb_3 mutant and in the Δaa_3 mutant was much less than in the wild type. Again, in the $\Delta aa_3/cbb_3$ mutant the succinate oxidation appeared to be unaffected by these inhibitors. More or less equal values of ascorbate/TMPD oxidation indicated the presence of cytochrome *c* oxidase(s) in the wild type, as well as in the Δcbb_3 and the Δaa_3 mutant. In the $\Delta aa_3/cbb_3$ mutant, the endogenous respiration was not enhanced at all upon the addition of ascorbate/TMPD, indicating that no cytochrome *c* oxidase was expressed.

Proton translocation

The proton-pumping capacity of the set of oxidase mutants was measured by using the oxygen-pulse method as described elsewhere (Raitio and Wikström, 1994; de Gier *et al.*, 1994). In the proton-translocation assay, succinate was used as the electron-donating substrate and either oxygen or potassium ferricyanide was used as the terminal electron acceptor (Table 2). The main goal was to study the proton-pumping capacity of the cbb_3-type cytochrome *c* oxidase. The H^+/e^- ratios of the Δaa_3 mutant and the $\Delta bb_3/aa_3$ mutant clearly show that the cbb_3-type cytochrome *c* oxidase does have the capacity to translocate protons. In Fig. 5, representative proton-translocation traces of the $\Delta bb_3/aa_3$ mutant are shown. The H^+/e^- ratios of the Δcbb_3 mutant and the $\Delta aa_3/cbb_3$

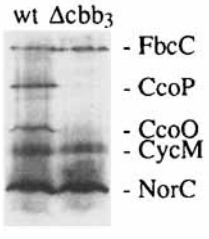

Fig. 4. SDS–PAGE and subsequent haem staining of membrane fractions of the wild type (wt) and the cytochrome cbb_3 mutant (Δcbb_3) of *P. denitrificans* after microaerobic cultivation. The *c*-type cytochromes indicated are subunits of the bc_1 complex (FbcC), subunits of cytochrome cbb_3 (CcoP, 45 kDa; CcoO, 30 kDa), cytochrome c_{552} (CycM), and a subunit of the NO reductase (NorC).

Table 1. Oxygen-consumption measurements of whole-cell suspensions of different *P. denitrificans* strains: Pd1222 (wild type), Pd92.20 (Δaa_3 mutant), Pd27.21 (Δcbb_3 mutant), and Pd93.12 ($\Delta aa_3/cbb_3$ mutant).

Strain	Substrate inhibitor	Endogenous –	Endogenous AA	Endogenous myx	Succinate –	Succinate AA	Succinate myx	Ascorbate/TMPD –
Wild type		1.5	0.9	0.9	2.4	0.6	0.5	3.7
Δaa_3 mutant		1.3	1.2	1.1	2.9	2.6	2.7	2.5
Δcbb_3 mutant		1.4	1.2	1.3	2.7	1.9	1.7	2.6
$\Delta aa_3/cbb_3$ mutant		2.2	2.2	2.2	2.1	2.1	2.1	0.0

Rates after addition of exogenous substrate have been corrected by subtraction of the corresponding endogenous rates. Values are the means of three independent assays (nmol O_2 per mg of protein per s). Inhibitors: antimycin A (AA), myxothiazol (myx).

mutant confirm that the aa_3-type cytochrome *c* oxidase and the bb_3-type quinol oxidase both translocate protons. Upon the addition of inhibitors of the bc_1 complex, the H^+/e^- ratio drops from 3 to 2 (not shown). This is consistent with the decreased ratio of $2H^+/e^-$ that is detected only in the $\Delta aa_3/cbb_3$ mutant. This suggests that ubiquinol apparently was preferentially oxidized by ubiquinol:cytochrome *c* oxidoreductase (bc_1 complex) rather than by ubiquinol:oxygen oxidoreductase (cytochrome bb_3).

With the artificial electron-donating couple ascorbate/TMPD, proton translocation was barely detectable in the absence of cytochrome aa_3. However, at elevated TMPD concentrations (final concentration: 2–3 mM) some proton translocation was observed, albeit never more than 50% of the theoretical maximum (not shown). The oxygen-consumption experiments indicate that, in the absence of cytochrome aa_3 and cytochrome cbb_3, no electron flow from ascorbate to oxygen is detectable in succinate-grown cells of *P. denitrificans*. Accordingly, no proton extrusion was observed with ascorbate/TMPD in the $\Delta aa_3/cbb_3$ mutant.

Discussion

P. denitrificans contains a respiratory network in which electrons are transferred from specific dehydrogenases to different types of terminal oxidases. One electron transfer route closely resembles the mitochondrial respiratory chain in which ubiquinol, reduced by either NADH or succinate, is oxidized by a supercomplex consisting of cytochrome *c* reductase (bc_1 complex), cytochrome c_{552} and cytochome *c* oxidase (cytochrome aa_3) (Berry and Trumpower, 1985; Trumpower, 1991). In addition, it has been demonstrated that *Paracoccus* expresses a distinct respiratory branch in which electrons are directed to a second terminal oxidase, the quinol-oxidizing cytochrome bb_3 (de Gier *et al.*, 1994).

Analysis of a recently generated $\Delta aa_3/bb_3$ mutant revealed that the aerobic respiration proceeds via an alternative cytochrome *c* oxidase (de Gier *et al.*, 1994). In the present study, the allocated gene cluster of this cbb_3-type oxidase has been cloned and sequenced. It has previously been demonstrated that no quinol oxidase activity remains in a Δbb_3 mutant (de Gier *et al.*, 1994), and here we show that a $\Delta aa_3/cbb_3$ double mutant does not

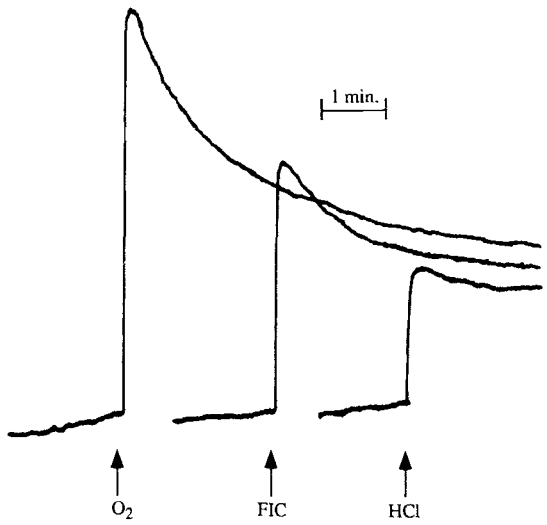

Fig. 5. Proton translocation in whole cells from strain Pd93.11 ($\Delta aa_3/bb_3$) with succinate as substrate. An anaerobic cell suspension was pulsed with equal volumes (10 µl) of air-saturated water (258 µM O_2), ferricyanide (1.0 mM FIC) and acid (1.0 mM HCl) to calibrate the system. The H^+/e^- ratio with oxygen is 3.0 and with ferricyanide the H^+/e^- ratio is 2.0.

Table 2. Proton-translocation measurements of whole-cell suspensions of different *P. denitrificans* strains.

Strain	Succinate O_2 (H^+/e^-)	Succinate $Fe(CN)_6^{3-}$ (H^+/e^-)
Wild type	2.93 ± 0.13	1.96 ± 0.09
Δaa_3 mutant	2.96 ± 0.15	1.94 ± 0.18
Δbb_3 mutant	2.97 ± 0.12	1.96 ± 0.12
$\Delta aa_3/bb_3$ mutant	2.95 ± 0.12	1.98 ± 0.16
Δcbb_3 mutant	3.01 ± 0.14	1.94 ± 0.13
$\Delta aa_3/cbb_3$ mutant	1.98 ± 0.11	1.97 ± 0.09

H^+/e^- ratios are averages ± standard deviation ($n = 8$). Cells were cultivated as aerobic batch cultures in minimal medium with succinate. The electron donor was succinate; anaerobic suspensions were pulsed with either oxygen (O_2) or ferricyanide ($Fe(CN)_6^{3-}$).

express cytochrome *c* oxidase activity (Table 1) (van der Oost *et al.*, 1995). Hence, it is concluded that, at least under a variety of tested conditions, these three types form the complete set of terminal oxidases expressed in *P. denitrificans*.

Characterization of cytochrome cbb_3

The products of the isolated *P. denitrificans ccoNOQP* gene cluster share a high degree of homology with the cytochrome *c* oxidase of *R. capsulatus* (Thöny-Meyer *et al.*, 1994) and the 'high-affinity' oxidase from several members of the Rhizobiaceae (Kahn *et al.*, 1993; Preisig *et al.*, 1993; Mandon *et al.*, 1994) (Fig. 3). Although the cbb_3-type oxidase apparently represents a distant member of the haem–copper oxidase family (van der Oost *et al.*, 1994; Saraste and Castresana, 1994), its cytochrome *b* subunit (CcoN) still shares some typical features with the catalytic subunit (subunit I) of these oxidases. Six histidines that are invariant in the available CcoN/FixN sequences have been aligned with the conserved histidine ligands of the redox centres of subunit I of cytochromes aa_3 and bo_3. The alignment shown in Fig. 3A suggests that H271, H321, and H322 are the most probable ligands of Cu_B, H409 of the penta co-ordinated haem B, and H411 and H122 of the hexa co-ordinated haem B in *P. denitrificans* CcoN.

Garcia-Horsman *et al.* (1994b) have recently reported a thorough biochemical analysis of the cbb_3-type oxidase from *R. sphaeroides* and have demonstrated the presence of a binuclear centre typical of haem–copper oxidases and the absence of Cu_A. Moreover, metal analysis revealed the ratio of haemC:haemB:copper to be 3:2:1. This agrees very well with the sequence data of cytochrome cbb_3: two haems B and a copper ion are located in subunit I (CcoN), and three haems C reside in the two cytochrome *c* subunits (one in CcoO and two in CcoP). Bosma (1989) has reported a 30 kDa cytochrome *c* associated with the alternative oxidase from *P. denitrificans* with an E_{m7} value of 322 mV. Gray *et al.* (1994) purified cytochrome cbb_3 from *R. capsulatus* and reported a 28 kDa cytochrome *c* (CcoO) with an E_{m7} value of 320 mV, a 32 kDa cytochrome *c* (CcoP) with an E_{m7} value of 265 mV, and a low-spin cytochrome *b* with an E_{m7} value of 385 mV. These data may indicate that CcoO is the direct electron donor of the redox centres (haems B and Cu_B) in CcoN. Indeed, the purified 2 subunit CcoNO complex (Fig. 1) retains TMPD oxidase activity. Apparently CcoP is less tightly bound to the cytochrome cbb_3 core and may play a role as electron carrier from the bc_1 complex to the CcoO–CcoN complex, which would be in agreement with the reported midpoint potential values.

Expression

Comparison of membrane proteins from the wild type and from the Δcbb_3 mutant of *P. denitrificans* by haem-stained SDS–PAGE (Fig. 4) identified the 30 kDa and 45 kDa cytochromes *c* as the CcoO and CcoP subunits of cytochrome cbb_3, respectively. Previously, Bosma (1989) performed a detailed analysis of the expression of *c*-type cytochromes in chemostat-grown cells of *P. denitrificans*, using the same haem analysis (reviewed by van Spanning *et al.*, 1995a). Bosma demonstrated that both the 30 kDa (CcoO) and the 45 kDa (CcoP) cytochromes *c* were abundantly present in membrane fractions when cells were grown in either nitrate-limited (anaerobically) or oxygen-limited (microaerobically) conditions. In succinate-limited (aerobic) cultures, on the other hand, the 45 kDa cytochrome *c* was absent and the 30 kDa cytochrome *c* was barely detectable (Bosma, 1989). It was concluded that the cbb_3-type oxidase plays an important role at decreased oxygen tensions.

In the promoter regions of all presently known *cco*/fixNOQP and *cco/fixGH*(*IS*) clusters is a sequence motif that closely resembles the binding site of the well-known anaerobic/microaerobic transcription activator FNR (Spiro and Guest, 1990). In *R. meliloti*, the 'oxygen state' is sensed by the haem protein FixL. In the absence of oxygen, this protein is autophosphorylated (FixL~P). Subsequently, the phosphate is transferred from the sensor to the regulator, FixJ, inducing its activation (FixJ~P). The latter modification results in transcription activation of *fixK*, the gene that encodes an FNR homologue. FixK, in turn, binds at the anaerobox upstream of the *fixNOQP* and *fixGHIS* clusters, thereby activating their transcription (David *et al.*, 1988; Batut *et al.*, 1989; Kahn *et al.*, 1989; Fischer, 1994). To date, no FixLJ system has been demonstrated in any non-nitrogen-fixing organism. However, a FixK-like protein, NNR, has been identified in *P. denitrificans* as a transcription activator of nitrite reductase and nitric oxide reductase, two polypeptide complexes that are involved in anaerobic respiration (van Spanning *et al.*, 1995b). Mutagenesis of the *nnr* gene, however, did not affect expression of cytochrome cbb_3. Here we show that in the flanking region of the *cco* locus of *Paracoccus*, a gene is located that potentially codes for a second transcription regulator: *fnrP* (Fig. 2A). Unlike NNR and FixK, this FNR homologue has an N-terminal cysteine cluster which, in the case of *E. coli* FNR, has been demonstrated to be involved in the binding of iron. The redox state of this iron ion determines whether the regulator is active or not (Spiro and Guest, 1990).

Energy conservation

Cytochromes aa_3 and bb_3 from *P. denitrificans* are proton-

pumping terminal oxidases (van Verseveld *et al.*, 1981; Solioz *et al.*, 1981; Puustinen *et al.*, 1989). Analysis of proton translocation in cell suspensions of the *Paracoccus* oxidase mutants offers the unique opportunity to measure proton translocation of individual oxidases in whole-cell suspensions. During succinate oxidation, a minimum H^+/e^- stoichiometry of 2 is theoretically expected when ubiquinol is oxidized directly by cytochrome bb_3 (Fig. 6). On the other hand, a ratio of $3H^+/e^-$ is the theoretical maximum when ubiquinol is oxidized via cytochrome bc_1 and cytochrome aa_3 (Fig. 6).

Measurements of the set of *P. denitrificans* mutants are in perfect agreement with the theoretical values (Table 2). Succinate oxidation by cytochrome bb_3 in the $\Delta aa_3/cbb_3$ mutant results in 2 H^+/e^-. In the conditions used for analysis of proton translocation (0.4–1.7 μM O_2), ubiquinol appears to be oxidized preferentially via the cytochrome *c* branch (bc_1 complex, cytochrome *c*, cytochrome *c* oxidase) rather than by the quinol oxidase cytochrome bb_3. This observation may be due to the low affinity for oxygen that has been measured for cytochrome bb_3 (K_m 10–20 μM O_2; de Gier, 1995). This may explain why, in the Δcbb_3 mutant, electrons mainly flow to cytochrome aa_3, resulting in a stoichiometry of $3H^+/e^-$. The proton pumping capacity of cytochrome cbb_3, as first reported by Raitio and Wikström (1994) in a Δaa_3 mutant, has been confirmed here with the $\Delta aa_3/bb_3$ mutant during electron transfer from succinate to oxygen (Table 2).

The conclusion that cytochrome cbb_3 does translocate protons, however, is in conflict with the previous measurements by de Gier *et al.* (1994). In the latter study an H^+/e^- stoichiometry of 2 has been measured during succinate oxidation of the $\Delta aa_3/bb_3$ mutant, suggesting that cytochrome cbb_3 does not pump protons. The main technical difference is that, in the experiment presented here, HEPES (0.5 mM) rather than glycylglycine (1.5 mM) has been used as buffer. The 'decoupling' of cytochrome cbb_3 in glycylglycine (de Gier *et al.*, 1994) has been confirmed by analysis of cells from a single batch in both buffers (not shown). The apparent buffer sensitivity of cytochrome cbb_3, a phenomenon that is not observed in the case of cytochromes aa_3 and bb_3, is not understood at present (see below).

The finding that all three terminal oxidases of *Paracoccus* (aa_3, cbb_3 and bb_3) couple the reduction of oxygen to the translocation of protons with maximal efficiency (1 H^+/e^-) also does not agree with analyses of growth efficiency in chemostat cultures of (wild-type) *P. denitrificans*, grown under a variety of conditions. The maximal growth efficiency is observed only under conditions in which cytochrome aa_3 is expressed (reviewed by Stouthamer, 1991). To address the discrepancy between the latter physiological studies and the above-described proton-pumping capacity of cytochrome cbb_3, we are currently cultivating a number of *Paracoccus* oxidase mutants under well-defined conditions. Preliminary data from a comparison of the growth yields of these mutants suggest that the energy-transducing efficiency of cytochrome cbb_3 is indeed lower than that of cytochrome aa_3 (de Gier, 1995).

The latter conclusion would be consistent with the aforementioned observation that the H^+/e^- stoichiometry of cytochrome cbb_3 is not a fixed number, and may be sensitive to, for example, buffer composition (de Gier *et al.*, 1994; Table 2). Moreover, proton pumping analysis of the $\Delta aa_3/bb_3$ mutant with ascorbate/TMPD as substrate, either in HEPES or in glycylglycine, results in H^+/e^- values that never exceed 50% of the ratio that is measured in the presence of cytochrome aa_3 (de Gier *et al.*, 1994; Raitio and Wikström, 1994). This apparent variability in the H^+/e^- ratio of cytochrome cbb_3 might be the result of certain structural deviations of subunit I within the regions that have been demonstrated to be important for the coupling between oxygen reduction and proton pumping (see below).

Residues involved in proton translocation

A major challenge of cytochrome oxidase research is elucidation of the proton-translocation mechanism at a

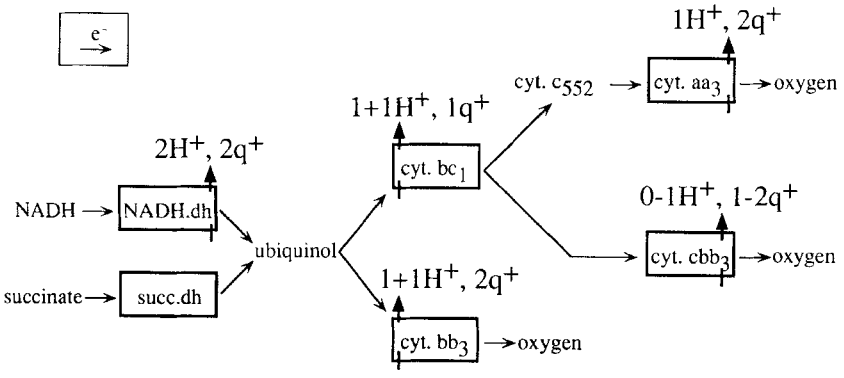

Fig. 6. Aerobic respiratory pathways in *P. denitrificans*. The proton-translocation ratios (H^+/e^-; protons per electron detectable in the medium) and the charge-separation ratios (q^+/e^-) for each of the contributing respiratory complexes are shown. NADH-dehydrogenase is represented by NADH.dh, succinate dehydrogenase by succ.dh, cytochrome reductase by cyt. bc_1, cytochrome c_{552} by cyt. c_{552}, aa_3-type cytochrome *c* oxidase by cyt. aa_3, cbb_3-type cytochrome *c* oxidase by cyt. cbb_3, and bb_3-type quinol oxidase by cyt. bb_3.

molecular level. This requires the identification of residues that are involved in the transfer of consumed (scalar) and/or pumped (vectorial) protons. Among the highly conserved residues in subunits I of cytochrome aa_3 and cytochrome bo_3, a large number of polar residues have been substituted by means of site-directed mutagenesis (reviewed by Hosler *et al.*, 1993). An aspartic acid in the domain between transmembrane helices II and III (loop II–III) has recently been proposed to participate in the influx of protons into the oxidase core (Garcia-Horsman *et al.*, 1995; Fetter *et al.*, 1995). Substitution of this conserved aspartate with an asparagine (D124N; numbering for *P. denitrificans* cytochrome aa_3, CtaDII, is used throughout) in both cytochromes aa_3 and bo_3 results in decoupling of proton pumping from electron transfer. In addition, substitution of two conserved asparagine residues (N113, N131) results in a decrease in the H^+/e^- ratio. Interestingly, proton pumping is recovered in a double mutant of *E. coli* CyoB, D124N/N131D (Garcia-Horsman *et al.*, 1995). It has been suggested that this cytoplasmic loop plays an important role as part of the entry to the pumped proton pathway, and moreover, that there should be a second channel, the chemical proton pathway. Residues that may be part of the latter pathway are a tyrosine (Y280) in helix VI, as well as two threonines (T344, T351) and a lysine (K354) in helix VIII. Non-polar substitutions for each of these residues result in a severe loss of oxidase activity (Hosler *et al.*, 1993). Both T351 and K354 have been proposed to participate in a proton-conducting channel towards the binuclear centre (Hosler *et al.*, 1993; Fetter *et al.*, 1995). Spectroscopic analyses suggest that T344 is in close proximity to the binuclear centre. Y280 may be a ligand to Cu_B, at least during part of the catalytic cycle of oxygen reduction (Hosler *et al.*, 1993). In addition, one of the invariant histidines that has been identified as a Cu_B ligand has recently been proposed to play a key role in the proton-translocation machinery. In the 'histidine cycle' model, a Cu_B co-ordinating histidine has been proposed to move back and forth in the binuclear 'pocket', thereby passing two protons from the inside to the outside (Morgan *et al.*, 1994).

Analysis of the recently resolved structure of *P. denitrificans* cytochrome aa_3 (Iwata *et al.*, 1995) supported many of the conclusions that were based on previous studies of the *E. coli* and *R. sphaeroides* oxidases (Hosler *et al.*, 1993; Fetter *et al.*, 1995; Garcia-Horsman *et al.*, 1995). Indeed, two suggested proton channels have been envisaged in the structural model (Iwata *et al.*, 1995). A pathway for consumed protons, from the cytoplasmic surface to the oxygen-binding site, appears to be made up of S291, K354, T351, the hydroxyl group of haem a_3, and, ultimately, Y280, which may donate a proton to oxygen (Fig. 3A, Table 3). With the exception of K354, all residues in this channel appear to be connected by hydrogen bonds, either directly or via putative solvent molecules. A second series of hydrophilic residues may constitute the pathway for pumped protons. A gate is formed by D124, N199, and T203; subsequently, a number of hydrophilic residues, again with additional putative solvent molecules, may form a pathway to E278 (Table 3). Beyond E278 the proton pathway is less clear. However, as proposed before (Morgan *et al.*, 1994), the structural model suggests that one of the histidine ligands, H325, may not be in a fixed position, but rather may switch between different conformational states. One option is that two subsequent protons, approaching the catalytic site via E278, convert the H325 imidazolate, via imidazole, into imidazolium. In the latter state, the positively charged H325 moves away from the binuclear centre, and no longer ligates Cu_B. The two protons of H325 imidazolium would leave the system via the exit pathway, in which groups of the haem a_3 molecule and/or residues in the periplasmic loop XI–X may be involved (Table 3), and H325 imidazole would return to its original position (for details, see Iwata *et al.*, 1995).

As cytochrome cbb_3 has the capacity to pump protons (Raitio and Wikström, 1994; Table 2), comparison of its sequence with that of the much better characterized cytochrome aa_3 is of particular interest. From the alignment presented in Fig. 3A, it is obvious that the sequences from CcoN/FixN show some significant deviation from both the aa_3-type and the bo_3-type oxidases, especially

Table 3. Comparison of residues proposed to be involved in the chemical and the pumped proton channels in cytochrome aa_3 (Hosler *et al.*, 1993; Fetter *et al.*, 1995; Iwata *et al.*, 1995), with counterparts in cytochrome cbb_3, based on the alignment presented in Fig. 3.

aa_3	Location	cbb_3	Consensus
Chemical channel			
a_3-hydroxyl	Haem	No hydroxyl	–
Y280	TM-VI	G	–
T351	TM-VIII	S	+
K354	TM-VIII	G	–
S291	TM-VI	M	–
Pump channel			
D399	Loop-IX/X	N	+
L393-carbonyl	Loop-IX/X	M	+
a_3-propionate	Haem	b_3-propionate	+
a_3-formyl	Haem	No formyl	–
H325	TM-VII	H	+
E278	TM-VI	A	–
N113	TM-II	X	–
N131	Loop-II/III	X	–
N199	TM-IV	V	–
T203	TM-IV	T	+
D124	Loop-II/III	X	–

In transmembrane helix II (TM-II) and the interconnecting loop between helices II and III (loop II–III) a reliable alignment is not possible; in this case the cbb_3 residues are marked 'X'.

with respect to the residues that have been predicted to be part of either one of the two proton channels (Table 3). Although alignment of the oxidase sequences at their N-termini is difficult, it is clear that the invariant aspartate residue in loop II–III of cytochrome *aa*$_3$ is not conserved in cytochrome *cbb*$_3$ (Table 3). In helix VI of cytochrome *cbb*$_3$, the tyrosine (Y280) and the glutamate (E278) that are conserved in all *aa*$_3$- and *bo*$_3$-type oxidases (and appear to be essential in the aforementioned proton pump model) are substituted by a glycine and an alanine, respectively. In helix VIII, the threonines are conserved as serines, but a glycine is found at the position of the conserved lysine (Fig. 3A, Table 3). In conclusion, comparison of the primary structures suggests that many residues that have been proposed to constitute the chemical and pumped proton channels in cytochrome *aa*$_3$ (and probably also in cytochromes *bo*$_3$ and *bb*$_3$) are not conserved in cytochrome *cbb*$_3$. These proton-conducting pathways are undoubtedly an essential component of the proton-translocation machinery. Hence, it is concluded that the design of the proton pump in cytochrome *cbb*$_3$ differs significantly from that present in the other oxidase types. Although site-directed mutagenesis would provide more definitive evidence, it is tempting to assume that these deviations in cytochrome *cbb*$_3$ might correlate with its apparently variable H^+/e^- coupling.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The strains of *P. denitrificans* and *E. coli* as well as the plasmids used in this study are listed in Table 4. Cells of wild-type *P. denitrificans* (Pd1222) and mutants were cultivated in aerobic batch cultures (0.5 l bottles with 100 ml of culture, on a rotary shaker at 30°C), with either brain–heart infusion (BHI) broth or minimal medium supplemented with 25 mM succinate, as described previously (van Spanning *et al.*, 1990). For microaerobic cultivation, cultures were not shaken. *E. coli* strains were cultivated in YT medium at 37°C. When appropriate, antibiotics were added: rifampicin (Rif, 40 mg l^{-1}), streptomycin (Sm, 50 mg l^{-1}), tetracycline (Tc, 12.5 mg l^{-1}), kanamycin (Km, 50 mg l^{-1}) and ampicillin (Amp, 100 mg l^{-1}).

Purification of the cbb$_3$*-type cytochrome* c *oxidase from* P. denitrificans

Cells from succinate-grown Pd93.11 (*Δaa*$_3$/*bb*$_3$) were harvested in the late log phase, and resuspended in 20 mM Bis-Tris propane (pH 7.3), 2 mM EDTA and 0.5 mM PMSF. Membranes were prepared by passage of the cell suspension twice through a French Pressure Cell and subsequent centrifugation (1.5 h, 150 000 × *g*). Membranes were solubilized in 20 mM Bis-Tris propane (pH 7.3), with 1.0% (w/v) dodecyl maltoside and 0.15 mM PMSF (30 min, 4°C). The suspension contained approx. 10 mg protein per ml and was cleared by ultracentrifugation (30 min, 150 000 × *g*). The solubilized membrane proteins were applied to a Q-Sepharose HP (Pharmacia) column in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was eluted with a linear NaCl gradient (0.0–1.0 M). Oxidase activity was measured spectrophotometrically with TMPD as described previously (Lübben *et al.*, 1994). Fractions with cytochrome *c* oxidase activity eluted at 0.3 M NaCl. Pooled fractions were applied onto a sucrose gradient, as described previously (Gray *et al.*, 1994). Fractions with TMPD oxidase activity were applied to a Chelating–Sepharose FF (Pharmacia) column, saturated with Cu^{2+}. A linear ammonium chloride gradient (0.0–1.0 M) in 20 mM Tris (pH 8.0), 0.5 M NaCl, 15 mM PMSF and 0.03% (w/v) dodecyl maltoside was used to elute bound proteins. Cytochrome *c* oxidase activity eluted at 0.3 M ammonium chloride. As a last purification step the active fractions were loaded onto a Q-Sepharose HP column (Pharmacia) in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was

Table 4. Strains and plasmids.

Strain/Plasmid	Relevant characteristics	Reference/Source
Strain		
E. coli		
TG1	*supE hsdD5 thi* Δ(*lac*$^-$ *proAB*) F′ (*traD36 proAB lacI*^{q} *lacZΔM15*)	Sambrook *et al.* (1989)
HB101	F$^-$ *hsdS20* ($r_B^- m_B^-$) *lacY1 proA2 recA13*	Boyer *et al.* (1969)
S17.1	Sm^R *pro* ($r_B^- m_B^+$) RP4-2, integrated (Tc::Mu) (Km::Tn*7*)	Simon *et al.* (1983)
P. denitrificans		
Pd1222	Rif^R, enhanced conjungation frequencies, ($r_B^- m_B^+$)	de Vries *et al.* (1989)
Pd92.20	Pd9218 derivative, Δ*ctaDI* Δ*ctaDII*	de Gier *et al.* (1994)
Pd26.21	Pd1222 derivative, *qoxB*::Km^R	de Gier *et al.* (1994)
Pd93.11	Pd9220 derivative, Δ*ctaDI* Δ*ctaDII cyoB*::Km^R	de Gier *et al.* (1994)
Pd27.21	Pd1222 derivative, *ccoNO*::Km^R	This work
Pd93.12	Pd9220 derivative, Δ*ctaDI* Δ*ctaDII ccoNO*::Km^R	This work
Plasmid		
pUC19	Amp^R *lacZ′*	Yanisch-Perron *et al.* (1985)
pUC4KIXX	Km^R (Tn*5*)	Pharmacia
M13mp18/19	pUC18/19 mcs, *lacZ′*	Sanger *et al.* (1980)
pRK2020	Tc^R pRK2013 Km::Tn*10*	Ditta *et al.* (1985)
pGRPd1	*oriV* (colE1) Amp^R *oriT* Sm^R (Tn*1831*)	van Spanning *et al.* (1990)
pCco1	pUC19 derivative, *ccoNOQP*	This work
pCco2	pUC19 derivative, *ccoNAorfN*	This work
pCco3	pUC19 derivative, *ccoNOQPGH′*	This work
pCcoNO::Km^R	pCco1 derivative, *ccoNO*::Km^R	This work
pRTd27.21	pGRPd1 derivative, *ccoNO*::Km^R	This work

eluted with a linear NaCl gradient (0.0–1.0 M). Active fractions eluted at 0.3 M NaCl. The purified oxidase was analysed by SDS–PAGE and spectrophotometrically (Laemmli, 1970; de Gier *et al.*, 1994). Haem staining was performed as described by Thomas *et al.* (1976).

DNA manipulation

General cloning techniques were carried out as described by Ausubel *et al.* (1993). Conjugations were performed as described previously (de Vries *et al.*, 1989). The matings of *Paracoccus* host strains were performed either directly with an *E. coli* S17.1 derivative that carried the plasmid of interest or via a triparental mating using any *E. coli* strain transformed with the plasmid of interest, in combination with *E. coli* HB101(pRK2020) containing the 'helper plasmid'. Mutagenesis was performed using pGRPd1 as described by van Spanning *et al.* (1990).

The PCR was performed using Super-Taq polymerase (HT Biotechnology Ltd.), essentially as described by de Gier *et al.* (1994). Primers were synthesized according to the amino acid sequence of the N-terminal peptide of the 30 kDa subunit of the *Paracoccus* cbb_3 complex (CcoO) (nos 171 and 172). These were degenerate primers with a bias for G:C at the third position of a codon. The sequences of these primers are as follows: 171 (sense), 5′-AAAAAAGTCTGCNAT[T/C/A]CTNGA[A/G]CA[T/C]-3′; and 172 (antisense), 5′-AAAAGAATTC[A/G/T]ATNCGNGNAACNACNAG[A/G]AA-3′.

DNA sequencing was performed using the dideoxy method (Sanger *et al.*, 1977) on fragments subcloned into M13mp18/19 (Sanger *et al.*, 1980) with the *Taq* dye dideoxy terminator cycle sequencing kit, and the *Taq* dye primer cycle sequencing kit (Applied Biosystems). Sequence analysis was carried out on a 370A DNA sequencer (Applied Biosystems). Editing of the sequences was performed with the SEQUENCHER 2.1 Program (Gene Codes Corp.).

Oxygen-consumption analysis

Oxygen consumption by bacterial cell suspensions was performed polarographically with a Clark-type oxygen electrode, as described by de Gier *et al.* (1994).

Proton translocation

Proton translocation was studied by using the oxygen-pulse method in intact cell suspensions (de Gier *et al.*, 1994; Raitio and Wikström, 1994). Cells were harvested in the late log phase from succinate batch cultures, washed twice and resuspended in 150 mM KCl (final OD_{660} is 100), and diluted four times in reaction medium (pH 7.4) in the stirred anaerobic reaction vessel (3.0 ml). The buffer contained 100 mM KCl, 100 mM KSCN, 0.5 mM HEPES (pH 7.4) and 30 μM rotenon, as described by Raitio and Wikström (1994). Succinate was used as the electron donor (final concentration 2.5 mM). The reactions were started with small pulses (5–20 μl) of air-saturated water (containing 258 μM O_2 at 25°C) or 1.0 mM potassium ferricyanide, made anaerobic with argon. Pulses were calibrated with 1.0 mM HCl or 0.5 mM oxalic acid, made anaerobic with argon. Controls with the protonophore FCCP were routinely included to ascertain if the observed proton ejection was due to proton translocation.

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